Journal of Experimental Botany, Vol. 55, No. 401, pp. 1325–1333, June 2004
DOI: 10.1093/jxb/erh153 Advance Access publication 7 May, 2004

Journal of Experimental Botany

RESEARCH PAPER

Expression analysis of a chicory fructan 1-exohydrolase gene reveals complex regulation by cold

An Michiels[1,*], Andre Van Laere[1], Wim Van den Ende[1] and Mark Tucker[2]

[1] *Laboratory for Molecular Plant Physiology, Institute of Botany and Microbiology, K.U. Leuven, Kasteelpark Arenberg 31, B-3001 Leuven, Belgium*

[2] *United States Department of Agriculture, Agricultural Research Service, Soybean Genomics and Improvement Laboratory, Building 6, BARC-West, 10300 Baltimore Ave., Beltsville, MD 20705-2350, USA*

Received 14 January 2004; Accepted 15 March 2004

Abstract

The gene for a recently identified cDNA, 1-FEH IIa, encoding a fructan 1-exohydrolase was isolated and cloned from *Cichorium intybus* and a 1149 bp promoter fragment was characterized. An analysis of the genomic 1-FEH IIa sequence indicated that the gene (*FEHIIa*) consists of six introns and seven exons, which is similar to plant invertase genes. Like invertase genes, *FEHIIa* also contains the 9 nt mini-exon encoding the tripeptide DPN. A database search for *cis*-acting response elements within its promoter identified multiple elements that appear to have relevance to cold-induced expression of the gene in field-grown roots. Promoter analysis by transient expression assay demonstrated that the *FEHIIa* gene promoter is highly expressed in etiolated *Cichorium* leaves and cold-stored roots, which correlated well with the high level expression detected by RNA blot analysis. Cold also enhanced *FEHIIa* reporter gene expression in green leaves, however, the reporter gene activity was much lower compared with similar induction experiments in etiolated leaves. Promoter deletion analysis demonstrated the presence of potential cold-responsive ABRE and/or CRT/DRE elements in the –22 to –172 region, while regions –933 to –717 and –493 to –278 contain elements that can down-regulate expression at the conditions used. Characterization of the *FEHIIa* promoter may provide tools to study cold-induced expression and to increase freezing tolerance in agricultural crops.

Key words: Cold induction, fructan, fructan exohydrolase, gene structure, promoter analysis.

Introduction

The most prominent storage carbohydrate in the plant kingdom is starch; nevertheless, fructan (a fructose polymer) is used as a storage compound in approximately 15% of flowering plant species (Hendry, 1993; Cairns *et al*., 2000). Unlike starch, fructans are water-soluble and are believed to be localized in the vacuole (Frehner *et al*., 1984; Wiemken *et al*., 1986). Fructans occur in many economically important crops, for example, asparagus, onion, chicory, wheat, rye, oats, and grasses, but are absent in species such as tobacco, potato, and *Arabidopsis*. The focus of the current study is the inulin-type of fructans that consist of linear β (2→1) linked fructofuranosyl units found in Asteraceae species such as chicory, thistle, and dandelion (Lewis, 1993; Wiemken *et al*., 1986).

Inulins are widely used in food as healthy alternatives for low-calorie sweeteners, dietary fibre, and fat substitutes (Fuchs, 1991). It has been suggested that the human intestinal flora is improved by a daily intake of low amounts of inulin that stimulate the growth of Bifidobacteria and Lactobacilli in the colon (Roberfroid *et al*., 1998; Taper *et al*., 1999; Yamamori *et al*., 2002). During the past decade, industrial fructan production has increased from 1 kiloton to 100 kilotons annually. However, further scaling up of inulin production from chicory is hampered by *in vivo* expression of fructan 1-exohydrolase activity (1-FEH; EC 3.2.1.80) that degrades

* Present address and to whom correspondence should be sent: Department of Cell Biology and Molecular Genetics, University of Maryland, HJ Patterson Building 073, College Park MD 20742, USA. E-mail: michiels@wam.umd.edu

Abbreviations: ABA, abscisic acid; ABRE, abscisic acid responsive element; COR, cold response; CRT/DRE, C repeat/dehydration responsive; FEH, fructan 1-exohydrolase; GUS, b-glucuronidase; HE-TAIL, high efficiency thermal asymmetric interlaced; Luc, luciferase.

inulin and limits its yield (Van den Ende and Van Laere, 1996; Van den Ende *et al*., 1996). A better understanding of the molecular regulation of 1-FEH expression may provide important information and tools necessary to increase inulin yield.

Three fructanase cDNAs (1-FEH I, 1-FEH IIa, and IIb) have been identified in *Cichorium intybus* (Van den Ende *et al*., 2001). Enzyme activity for 1-FEH I was ascertained by heterologous expression in potato, and the identity of all three cDNA clones were confirmed by comparison with trypsin digest and mass spectrometry of the purified enzymes (Van den Ende *et al*., 2000, 2001). Sequence comparison of 1-FEH I to 1-FEH IIa and IIb reveals 50% sequence identity. The cDNAs for 1-FEH IIa and IIb are closely related sharing 94% sequence identity at the amino acid level. Expression studies by Van den Ende *et al*. (2001) indicated that 1-FEH I is highly expressed in stored roots while 1-FEH II was most abundant in etiolated leaves. However, De Roover *et al*. (1999) demonstrated that defoliation caused a rapid increase in 1-FEH II expression in roots suggesting that 1-FEH II may be induced as a survival enzyme when energy demands increase and fast regrowth is necessary.

Derived amino acid sequences for the 1-FEH cDNAs reveal a number of highly conserved motifs shared in invertases, including the potential active site motif WEC(V/P)D and the β-fructofuranosidase motif DPN. Genes for invertases have been isolated from carrot (Sturm, 1996), potato (Maddison *et al*., 1999), *Arabidopsis* (Haouazine-Takvorian *et al*., 1997), maize (Xu *et al*., 1996), and tomato (Elliott *et al*., 1993). The structure of the invertase genes are fairly similar, each containing between six and eight exons (Tymowska-Lalanne and Kreis, 1998). Most of the genes contain an extremely small exon (exon II) that encodes the highly conserved tripeptide DPN, one of the smallest exons published so far. Bournay *et al*. (1996) demonstrated alternative splicing of this mini-exon in potato invertase genes during cold induction and proposed a physiological role for the deletion of the β-fructofuranosidase DPN motif. Interestingly, since fructans are proposed to be localized in the vacuoles, it might be expected that plant 1-FEH cDNAs would be more closely related to the vacuolar invertases; however, unexpectedly, based on sequence alignments, 1-FEH is more similar to the cell wall invertases than to vacuolar invertases (Van den Ende *et al*., 2000, 2001).

Low temperatures are a major environmental factor affecting plant growth and crop productivity. Plants can acquire increased frost-tolerance after a period of exposure to low, non-lethal temperatures through a complex process called cold acclimation (Levitt, 1980). The cold–hardening process is associated with changes in gene expression that result in several biochemical and physiological changes including alterations in lipid, protein, and carbohydrate composition (reviewed in Warren, 1998). Membranes are the primary targets of both freezing and desiccation injuries in cells (Oliver *et al*., 2001). Recent reports indicate that fructans might have a membrane protective effect during stress conditions (Demel *et al*., 1998; Livingston and Henson, 1998; Hincha *et al*., 2000). Moreover, the fructan content of Canada thistle and dandelion roots affected freezing temperature tolerance and played a role in herbicide-based weed control (Wilson and Michiels, 2003).

Although the purification and characterization of fructan 1-exohydrolase enzymes and cDNAs from plants have been reported (Claessens *et al*., 1990; De Roover *et al*., 1999; Van den Ende *et al*., 2000, 2001), very little is known about the structural organization of the genes or their gene promoters. Moreover, the molecular relationship and evolutionary origin of plant 1-FEH and β-fructofuranosidases, in general, still remain to be elucidated. The identification and structural organization of the *FEHIIa* gene in *Cichorium intybus*, and the low-temperature-regulation of a 1149 nt *FEHIIa* promoter fragment in a transient expression assay are reported here.

Materials and methods

Plant material

Chicory (*Cichorium intybus* var. *foliosum* cv. Flash) was planted on 18 May 1998 in a local field in Belgium with sandy, loamy soil. Field-grown chicory roots were collected weekly between 10 September and 5 November. Afterwards, on 8 November, plants were uprooted, defoliated, and stored for 3 weeks at 1 °C. While in storage, root samples were taken on 10 and 16 November.

For all the other results, chicory was grown in a greenhouse and 3-month-old plants used for all the experiments. To test the influence of cold storage, 3-month-old roots were uprooted, defoliated and stored at 4 °C for 2 weeks. Etiolated chicory leaves were grown and harvested as described by Van den Ende *et al*. (1996).

Isolation of DNA and RNA

The indicated plant organs from chicory were harvested, immediately immersed in liquid nitrogen, and temporarily stored at –80 °C. DNA was extracted from etiolated leaves using the optimized extraction method described by Michiels *et al*. (2003a). RNA from 1 g of frozen tissue was extracted using an RNA extraction kit (TRIREAGENT, MRC Inc., USA).

Northern blot hybridization

Total RNA (10 μg) was denatured in 12.55 M formamide, 2.2 M formaldehyde and 20 mM MOPS buffer (pH 7.2), containing 5 mM Na-acetate and 0.1 mM EDTA at 65 °C for 5 min. Samples were separated by electrophoresis (8–10 V cm^{-1}) in a 1.2% (w/v) agarose gel containing 2.2 mM formaldehyde in the same MOPS buffer. Subsequent northern blotting was performed by RNA transfer overnight to a Hybond-NX membrane (Amersham, Pharmacia Biotech, Piscataway, NJ, USA) via capillary blotting in 20× SSC buffer. The membrane was washed in 10× SSC buffer and RNA was cross-linked to the membrane with a UV-GS gene linker (150 mJ, BioRad). Prehybridization was performed for at least 2 h at 68 °C in 500 mM sodium phosphate buffer pH 7.2, 7% SDS, 1 mM EDTA. The labelled probe was then added to the hybridization buffer and

incubated overnight at 68 °C. The hybridized membrane was washed successively in 0.5× SSC 0.1% SDS, and 0.1× SSC 0.1% SDS and exposed to a phosphor-imager screen. Signals were quantified using Phosphor-Imager technology (Fuji, BAS 1000; Software PCBAS 2.09). As a test to confirm that nearly equal amounts of RNA were loaded into the gel and transferred to the membrane, the membrane was also hybridized with a labelled probe for an 18S rRNA from *Cichorium intybus*.

Genomic amplification

Gene-specific primers were designed based on 1-FEH IIa cDNA sequence information (Van den Ende *et al*., 2001) to PCR amplify the *Cichorium intybus FEHIIa* gene. ExI-F (5′-aaagtcatcgatccccaaacacaca-3′) and ExIII-R (5′-aacgttgctgcatacggattgtattg-3′) amplified a 1629 nt fragment, while primers ExIII-F (5′-ccagggagtgtaccatttcttctacca-3′) and UTR3-R (5′-ccaaacctactctttcaaccttcactgg-3′) yielded a 3003 nt fragment. Reaction mixtures (15 µl) contained 20 ng template DNA, 1.2 U KlenTaq polymerase (Sigma, St Louis, MI, USA), 1× PCR buffer, 1.1 mM $MgSO_4$, 0.2 mM dNTP, and 0.4 µM of each forward and reverse primer. The PCR mixture was subjected to the following PCR programme: one cycle at 94 °C for 5 min, five cycles at 95 °C for 30 s, 68 °C for 4 min, 30 cycles at 95 °C for 35 s, 65 °C for 4 min, with a final extension at 72 °C for 7 min. The samples were analysed by agarose gel electrophoresis. Amplification products were cloned into vector pGEM-T (Promega, Madison, WI, USA) and sequenced. A High Efficiency Thermal Asymmetric Interlaced PCR (HE-TAIL-PCR)-based approach was used to clone the 1149 nt promoter fragment of *FEHIIa* (Michiels *et al*., 2003*b*). After obtaining the sequence for the HE-TAIL-PCR product, two gene-specific primers –1149 *Hin*dIII-for (5′-GTAAGCTTCGCAGACCTCTATCCATATATTAGTTC-3′) and ATGKpnI-rev (5′-AAAGGTACCTCTTCATGATGAGTGTGTGTGTTTGG-3′) were synthesized to amplify a 1149 bp promoter fragment using a proofreading *Pfu* DNA polymerase (Promega, Madison, WI, USA) and the following PCR conditions: 1 cycle at 94 °C for 5 min, 5 cycles at 95 °C for 30 s, 67 °C for 1.5 min, and 30 cycles at 95 °C for 35 s, 65 °C for 1.5 min, with a final extension at 72 °C for 7 min. The promoter fragment was cloned into the pGEM-T vector (Promega, Madison, WI, USA) and M13 forward and reverse primers were used for sequencing.

Chimeric reporter gene fusions

A *Hin*dIII–*Eco*RI fragment containing the cauliflower mosaic virus (CaMV) 35S promoter, luciferase gene, and nopaline synthase terminator (NOS-ter) were cloned into the corresponding sites in pUC19 to create the pD0432 vector (Ow *et al*., 1986). The luciferase reporter gene was later modified to include a *Kpn*I restriction site at the 5′ end (Koehler *et al*., 1996). The *Hin*dIII–*Kpn*I restriction fragment of the *FEHIIa* promoter was cloned into the corresponding sites of the pD0432 vector after deleting the CaMV35S promoter to create the pFEHIIa-luc plasmid. The pBI221 vector (Clonetech, Palo Alto, CA, USA), which contains a CaMV35S promoter to express a β-glucuronidase (GUS) reporter gene, was used as an internal control during transient expression assays. The 933, 717, 493, 278, and 172 bp promoter fragments were obtained using a proofreading *Pfu* DNA polymerase (Promega, Madison, WI, USA), pFEHIIa-luc plasmid as template and the forward primers, –933 *Hin*dIII-for (5′-TTTAAAGCTTCAATTACGTTTTTACCATCAAAGA-3′), –717 *Hin*dIII-for (5′-ACTTAAGCTTCAAATTAGTGGTCAATGGTCTGTT-3′), –493*Hin*dIII-for (5′-AAACAAGCTTATGAATATGCCGTCATTTACTTATT-3′), –278*Hin*dIII-for (5′-AAAGAAGCTTAAAATGGGTCCTTTAAAGACAATGA-3′), and –172*Hin*dIII-for (5′-TTCAAAGCTTCGAAAGTTCAAGGGGCAATA-3′) in combination with reverse primer ATGKpnI-rev. PCR conditions were the same as described earlier to amplify the 1149 bp promoter fragment. The deletion fragments were digested with both *Hin*dIII and *Kpn*I and ligated into the corresponding restriction sites of pFEHIIa-luc to replace the 1149 bp promoter fragment and are named p1149-luc, p933-luc, p717-luc, p493-luc, p278-luc, and p172-luc, respectively. All constructs were verified by automated sequencing.

Transient expression assay

Etiolated leaf explant pieces were prepared by making 0.5 cm transverse sections from etiolated leaves and then three pieces were positioned upright on 1% agar Petri plates containing 50 µg ml^{-1} ampicillin. Root explants were prepared by cutting 1 cm thick sections from the upper part of 3-month-old roots. Leaf discs (2 cm) were cut from the green leaves of chicory. Root and green leaf discs were arranged centrally on the agar plate. Each particle bombardment session included four independent replica plates and each bombardment session was repeated at least once. Equal molar concentrations of FEHIIa-luciferase (pFEHIIa-luc) and 35S-GUS (pBI221) constructs were coprecipitated (0.2 pmol per bombardment) onto 1.6 µm gold carriers. The constitutively expressed pBI221 was used as an internal control to normalize transient expression data and to reduce variation between bombardment sessions (Koehler *et al*., 1996). A biolistic PDS-1000/He particle gun (Bio-Rad, Richmond, CA, USA) was used to bombard explants. Bombardment parameters included a helium pressure of 1350 psi, 1/4 inch distance between rupture disc and microcarrier, and 5 cm distance between stopping screen and Petri plate. An important parameter in these bombardment experiments is that the mole ratio and concentration of test constructs and the internal control construct is the same for each of the mixtures. To ensure equal ratios, plasmid DNA was quantified by agarose gel electrophoresis and by spectrophotometric analysis at 260 nm. Explants were incubated for 17 h in the dark at room temperature unless indicated otherwise. Temperature induction was performed by placing explants in the dark in incubators at 25 °C, 16 °C, or 4 °C for 17 h or in a freezer at –20 °C for 1–4 h with subsequent incubation at 16 °C for 16 h or 13 h, respectively. After 17 h incubation at the indicated temperature, thin 1 mm sections were sliced from the bombarded surfaces and frozen in liquid nitrogen. Protein was extracted from frozen tissue by homogenization (3 ml g^{-1} fresh tissue) in 0.1 M sodium phosphate, pH 7.8, 0.2% Triton X-100, 2 mM EDTA, 1 mM DTT, and 0.1% BSA. Fluorometric assays for GUS activity were performed as described by Jefferson *et al*. (1987) except that samples were heated to 55 °C for 30 min to reduce endogenous GUS-like activity (Tucker *et al*., 2002). Luciferase was assayed as described in the Luciferase Assay Guide (Analytical Luminescence Laboratory, San Diego, CA, USA) using a Bertold Luminometer (Pittsburgh, PA, USA). The photons emitted after injection of the luciferase substrate were integrated over a 10 s interval and expressed as relative light units per 10 s.

Sequencing and sequence analysis

The Big DyeTM Terminator Cycle sequencing Ready reaction kit and the model 337 automated sequencer (Perkin-Elmer Applied Biosystems, CA, USA) were used to sequence amplification product and constructs. Possible *cis*-regulatory elements were identified based on PLACE database analysis (http://www.dna.affrc.go.jp/htdocs/PLACE).

Results

1-FEH II induction in field-grown and cold-stored chicory roots

In field-grown plants, 1-FEH II mRNAs were absent for the first 143 d. On day 150, 1-FEH II is induced, but

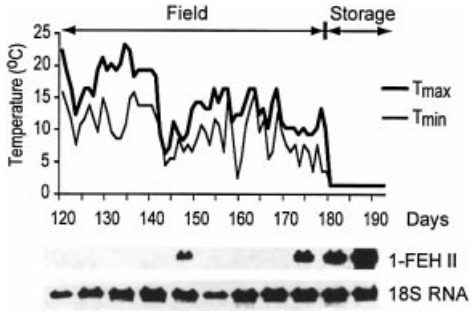

Fig. 1. RNA blot analysis of FEHIIa gene expression. Northern blot containing RNA from mature field-grown chicory roots. Roots for RNA extraction were collected weekly between 120 d and 180 d after planting. Plants were uprooted after 180 d and stored at 1 °C. Root samples were again taken weekly during cold storage. The lanes in the northern blot are aligned with the approximate root collection date on the temporal axis in the graph above. Maximum and minimum daily temperatures and the temperature in storage are displayed. RNA blots were probed with full-length cDNAs for 1-FEH IIa and 18S rRNA. Because of high sequence identity between 1-FEH IIa and IIb, the 1-FEH IIa probe cannot discern between the two transcripts. Daily temperatures for Vlaams-Brabant were registrated by the Koninklijk Meteorologisch Instituut (Maandbulletin, September–December 1998).

disappears again in the following weeks (Fig. 1). Although different factors might be responsible for the sudden 1-FEH II induction, the severe temperature drop at days 143 and 144 might have influenced the expression pattern. Furthermore, the 1-FEH II genes were highly expressed when the minimum temperature declined severely after day 170. The hybridization signal intensified further when the plants were uprooted and put into cold storage (Fig. 1).

Analysis of the genomic sequence of FEHIIa

A genomic fragment of 4769 nt (accession AY323935) was identified by sequencing multiple overlapping fragments as described in the Materials and methods. The structural map of the *FEHIIa* gene is displayed in Fig. 2. The intron/exon organization of *FEHIIa* is compared with the genomic structures of dicot invertase genes from carrot, tomato, potato, and *Arabidopsis*. *FEHIIa* is organized into seven exons and six introns with similar intron–exon positioning as the cell wall invertases *Atβfruct1*, *InvDc2*, and *InvDc3* and vacuolar invertases *InvDc4*, *InvDc5*, *InvLe31*, and *InvZmIvr1*. The *FEHIIa* gene contains a 9 nt mini-exon (exon II) encoding the tripeptide DPN of the W(I/M)NDPNG region. This mini-exon is conserved in most plant invertases, except for the carrot cell wall invertase *InvDc1* that has the DPN region incorporated in the major exon (Sturm, 1995). Of particular interest here is that the mini-exon in the cell wall invertase genes is followed by a short intron or, in the case of *InvDc1*, no intron at all. By contrast, the vacuolar invertases have much longer second introns that can exceed 1500 nt (*InvLe31*, Fig. 2). Interestingly, *FEHIIa* has a long second intron of 1089 nt which is more similar to the vacuolar invertases. Sequence data for the *FEHI*

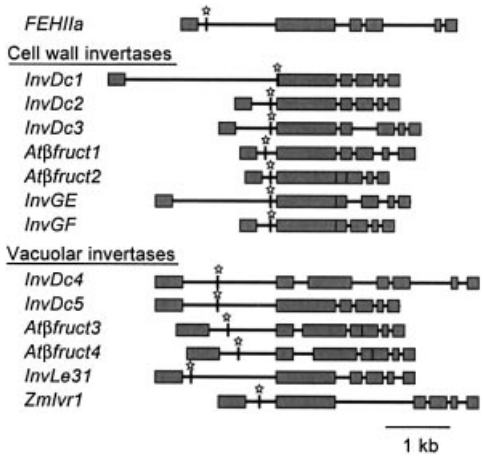

Fig. 2. Structural maps of genomic clones for FEHIIa (accession number AY323935), cell wall invertase from carrot (*InvDc1*, *InvDc2*, *InvDc3*; Sturm, 1996), potato (*InvGE*, *InvGF*; Maddison *et al*., 1999), and *Arabidopsis* (*Atβfruct1*, *Atβfruct2*; Haouazine-Takvorian *et al*., 1997) and vacuolar invertase from carrot (*InvDc4*, *InvDc5*; Sturm, 1996), *Arabidopsis* (*Atβfruct3*, *Atβfruct4*; Haouazine-Takvorian *et al*., 1997), maize (*ZmIvr1*; Xu *et al*., 1996), and tomato (*InvLe31*; Elliott *et al*., 1993). The coding regions are represented as darkened boxes with intervening non-coding sequences shown as lines. The mini DPN exons are highlighted with a star.

gene also indicates the presence of a long second intron (results not shown).

Sequence analysis of the FEHIIa *5′ upstream region*

The *Cichorium FEHIIa* upstream region was isolated by a HE-TAIL based amplification strategy and sequenced (Fig. 3). Van den Ende *et al*. (2001) identified a near full-length cDNA with 56 nt upstream of the ATG codon. A putative TATA box is located at –50 relative to the beginning of this cDNA (Fig. 3). A search for putative *cis*-acting elements in the promoter region was performed using PLACE, a database for plant *cis*-acting elements. Notably, motifs are found for response to auxin (ASF1, ARF, and CATATGGMSAUR), abscisic acid (ABRE), ethylene (GC box and ERE), gibberellin (GARE), and salicylic acid (ASF1 and W box). Two negative S1F elements are identified in the 933–717 region (Fig. 3). Motifs essential for glucose starvation-induced expression are also present in the *FEHIIa* 5′ upstream region including a GARE element, a GC box, a G box, and TATCCA elements. Conversely, SURE represents a sucrose-responsive element that is up-regulated by increasing sucrose supply. A possible Evening element and two adjacent CIRC elements required for circadian control of gene expression are also present. Moreover, two adjacent REalpha motifs, which play a role in phytochrome regulation, are detected in the *FEHIIa* promoter (Fig. 3).

Tissue specific expression of FEHIIa

Northern blot data for *FEHIIa* transcript accumulation indicated induction of the *FEHIIa* gene in etiolated leaves

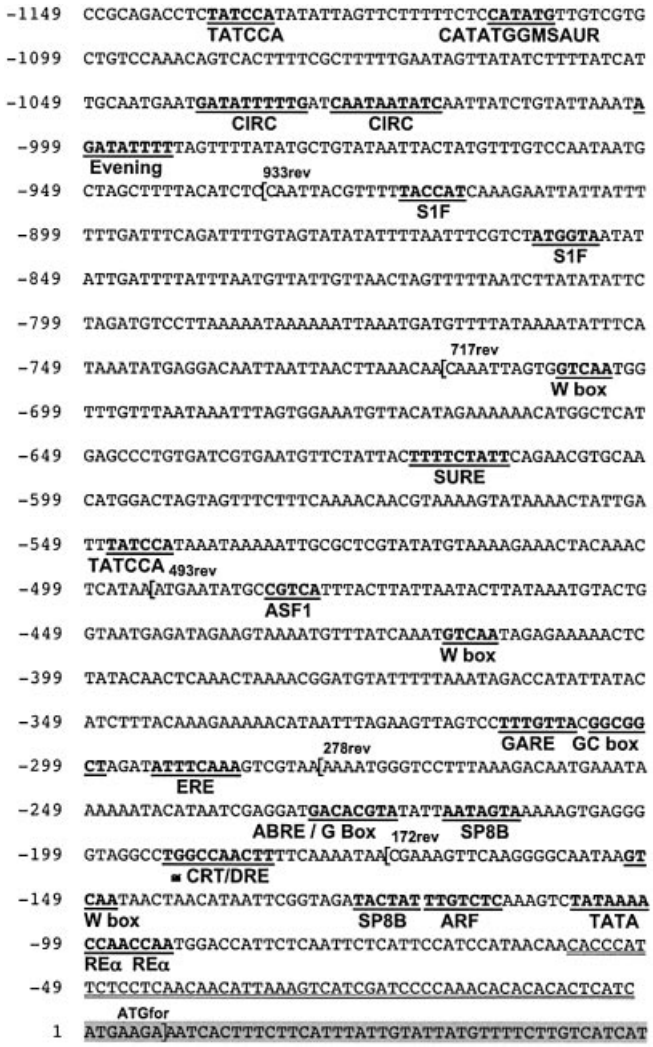

Fig. 3. Nucleotide sequence of the *FEHIIa* promoter and putative *cis*-acting elements. The nucleotides are numbered from the translation start site (+1). Brackets and numbers indicate the starting site of primers –933 *Hin*dIII-for, –717 *Hin*dIII-for, –493 *Hin*dIII-for, –278 *Hin*dIII-for, –172 *Hin*dIII-for, and ATGKpnI-rev. The beginning of the open reading frame is highlighted in grey, the 5′ UTR in the previously reported cDNA sequence (Van den Ende *et al*., 2001) is double underlined, and putative *cis*-acting elements are bold and underlined with the respective names listed under the element. Detailed descriptions for the noted *cis*-acting elements can be found in the PLACE database at http://www.dna.affrc.go.jp/htdocs/PLACE/. The gene sequence can be found with the accession number AY323935.

and cold-stored roots (Fig. 4). However, *FEHIIa* belongs to a small family of genes and mRNA blot analysis might also detect other isoforms (isoforms IIa and IIb share 94% identity). An alternative method to determine the relative expression level of *FEHIIa* in different plant tissues was used. The *FEHIIa* promoter region was fused to a luciferase reporter gene and introduced into different explant tissues by particle bombardment. A CaMV35S–GUS construct (pBI221) was used as an internal control. Reporter gene activity was measured in chicory roots,

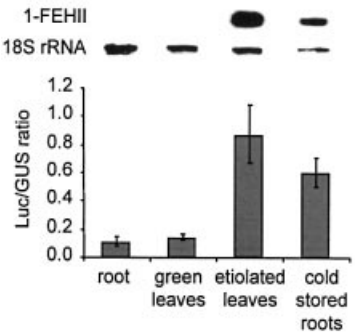

Fig. 4. Transient reporter-gene expression of pFEHIIa–luc constructs and FEHII northern blot analysis in different tissues of *Cichorium intybus*. Chicory explants were prepared from the roots of the greenhouse plants, green leaves, etiolated leaves, and cold-stored roots, and accumulation of FEHII mRNA was determine by northern blot analysis. Equivalent loading of RNA was confirmed with an 18S ribosomal probe. Equal molar concentrations of plasmids pFEHIIa-luc and pBI221 were co-precipitated onto gold particles and used for particle gun bombardment of explants. Reporter gene activity was measured in leaf and root explants after 17 h incubation at 25 °C for green leaves, etiolated leaves, and roots or 4 °C for cold-stored roots. Expression levels are indicated as the ratio of luciferase to GUS activity. Each error bar indicates the standard error of the mean for at least four independent particle bombardments of replica plates.

green leaves, etiolated leaves, and cold-stored roots after 17 h incubation at 25 °C for green leaves, etiolated leaves, and roots or 4 °C for the cold-stored roots. The normalized reporter gene activity for the *FEHIIa* promoter is highest in etiolated chicory leaves and cold-stored roots (Fig. 4). *FEHIIa* reporter gene expression correlates well with RNA blot analysis and indicates that the *FEHIIa* gene is induced in cold-stored roots.

Low-temperature-induced expression of FEHIIa

To investigate the effect of low temperature on *FEHIIa* expression further, transient expression assays were performed with roots, etiolated leaf explants, and green leaf discs exposed to several different temperature regimes. A 12–14-fold induction of luciferase expression is observed in root explants that were incubated for 1 h at –20 °C and then for 16 h at 16 °C. A very similar induction occurred in samples that were kept continuously at 4 °C after bombardment. Incubation at 4 °C for 1 h and 4 h, and subsequently at 16 °C for 16 h or 13 h, respectively, is responsible for a 3.5-fold and 4.2-fold increase compared with explants that were exposed continuously to 16 °C (Fig. 5). Incubation of root explants at –20 °C for 4 h results in a brown colouring of the tissue caused by freezing injuries of the cells. This severe treatment had a lethal effect on the bombarded root cells and no reporter gene activity could be measured. Similar induction patterns were observed in etiolated leaf explants after different temperature treatments (Fig. 5). Although the Luc/GUS ratios in green leaves were significantly lower, a 6-fold increase in reporter gene activity was observed in green leaves after a 17 h incubation at 4 °C.

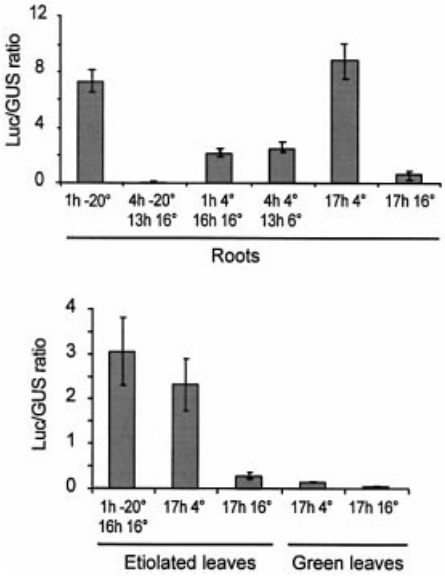

Fig. 5. Transient expression characteristics of the pFEHIIa-luc plasmid in roots and etiolated and green leaves exposed to different temperature regimes. Particle bombardment and reporting of expression levels are as described in Fig. 4. Explants were incubated at different temperatures (°C) after bombardment and analysed for reporter-gene expression. Each standard error represents at least four independent bombardments of replica plates.

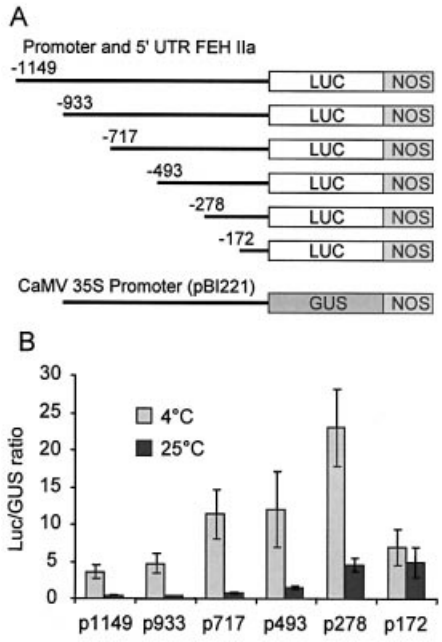

Fig. 6. 5′ deletion analysis of the *FEHIIa* promoter region. (A) The 5′ deletion constructs containing the *FEHIIa* upstream regions were ligated in a promoterless pD0432 luc plasmid. The numbers refer to the 5′ end in relation to the translation start site and also represent the respective names of the plasmids. (B) Equivalent amounts of plasmids p1149-luc, p933-luc, p717-luc, p493-luc, p278-luc, and p172-luc were introduced in 3-month-old root explants by particle bombardment with pBI221 (CaMV35S–GUS) as internal control. After bombardment samples were kept in the dark at 25 °C or 4 °C. Relative luciferase/GUS ratios are shown. Means and standard error bars represent replica plates of at least four independently bombarded samples. LUC, luciferase; NOS, nopaline synthase.

Deletion analysis

In order to characterize *cis*-elements that are critical to *FEHIIa* cold-induced expression, a series of 5′ deletion constructs were ligated into the pD0432 vector (Fig. 6A). Plasmids containing 5′ truncated promoter–luciferase constructs together with the pBI221(CaMV35S–GUS) internal control were cobombarded into root explants. The relative luciferase/GUS expression of the reporter genes was determined after 17 h in the darkness at 4 °C or 25 °C for each of the deletion constructs (Fig. 6B). Removal of the sequence to –717 in cold-induced explants caused an increase of about 3-fold in the luc/GUS ratio, while deletion of an additional 439 bp resulted in a 7-fold increase of reporter gene activity. Although a similar pattern was observed, the increase was considerably less in 25 °C incubated explants. Further deletion down to –172 decreased promoter activity in cold-induced explants, while activities were unaffected in the 25 °C incubated samples.

Discussion

The gene (*FEHIIa*) for a recently identified cDNA encoding a fructan 1-exohydrolase enzyme (1-FEHIIa) (Van den Ende *et al*., 2001) has been cloned and sequenced. In addition to a search for regulatory motifs in the gene promoter, the promoter has been partially characterized in a transient expression assay. A correlation was demonstrated between minimal daily temperature profiles and 1-FEH II mRNA expression patterns in field-grown chicory roots (Fig. 1). Genomic analysis of the *FEHIIa* gene indicated the presence of a tripeptide mini-exon, DPN, and indicated that the intron–exon organization resembles those of vacuolar invertase genes (Fig. 2). The 5′ upstream region of *FEHIIa* was successfully isolated from *Cichorium intybus* using a HE-TAIL-based method. Detailed analysis of the region using the PLACE database identified the presence of several putative *cis*-acting elements (Fig. 3). The expression patterns of the *FEHIIa* promoter–luciferase construct clearly demonstrate that *FEHIIa* is highly expressed in cold-stored root explants, suggesting a cold-specific induction, which correlates well with northern blot results (Figs 5, 6).

A detailed study of the genomic organization of *FEHIIa* and vacuolar invertase genes indicated that the intron–exon patterns of these enzymes are highly similar (Fig. 2). However, sequence alignments of β-fructofuranosidase protein sequences indicate that fructan 1-exohydrolases are more similar to cell wall invertase than to vacuolar invertase sequences (Van den Ende *et al*., 2001). It is noteworthy that cell wall invertases have basic p*I*s and are positively charged to form ionic bonds with apoplastic

components, while fructan 1-exohydrolases and vacuolar invertases have acidic p*I*s suggesting that they occur as soluble enzymes (Goetz and Roitsch, 1999). The conserved intron/exon organization for fructan 1-exohydrolases and invertases reinforce the idea of a common ancestral origin for both gene groups. Exon III is split by an intron in the *Atβfruct3* and *Atβfruct4* genes, while intron 4 is absent in both vacuolar genes. Moreover, extracellular invertase genes *InvDc1* and *Atβfruct2* also have some introns missing. However, Lambowitz and Belfort (1993) suggested that introns may be considered as mobile genetic elements during the course of evolution. It is suggested that gene duplication, intron skipping and mobility might have played an important role during the evolution of extracellular invertase, vacuolar invertase, and fructan 1-exohydrolase genes that differ in enzymatic properties, transcriptional regulation, tissue-specific and developmental expression. Therefore, because of the dichotomy between peptide similarities and gene structure, it may be postulated that during invertase gene evolution intron skipping and/or shifting occurred after fructan 1-exohydrolase and invertase diverged.

Like most plant β-fructofuranosidase genes, *FEHIIa* contains the tripeptide exon DPN, which might have important functions in enzyme conformation and catalytic activity (Tymowska-Lalanne and Kreis, 1998). Bournay *et al*. (1996) described DPN-exon alternative splicing in potato invertase genes during cold induction, indicating a putative role in post-transcriptional regulation of the gene. Recently, several publications illustrate post-transcriptional regulation of gene expression by alternative splicing (reviewed in Lorkovic *et al*., 2000). Although Bournay *et al*. (1996) described DPN-exon skipping in potato cell wall invertase genes during cold induction, alternative splicing of the *FEHIIa* gene was not observed by RT-PCR analysis of cold-stored chicory roots (results not shown). Moreover, the *FEHIIa* intron 1 sequence includes a possible branchpoint sequence CTAAC with an adjacent 15 nt U-rich region localized 72 nt from the 3′ end of the splice site for intron 1 that resembles the mini-exon splice signal organization in plant invertase genes (Simpson *et al*., 2000). The author also concluded that a long polypyrimidine tract (>8 nt) and a close positioning of the tract to the branchpoint sequence are important for efficient mini-exon splicing (Simpson *et al*., 2002). Thus, the structure of the *FEHIIa* gene suggests that it has all the features necessary for efficient splicing of the mini-exon.

The expression patterns of the *FEHIIa* promoter–luciferase construct clearly demonstrate that *FEHIIa* is more highly expressed in etiolated leaves as compared with green leaves of chicory explants and in cold-stored roots as compared with roots stored at room temperature (Fig. 4). Moreover, transient expression results indicated that *FEHIIa* expression is greatly enhanced in etiolated leaves exposed to cold treatments (Fig. 5). Progressive deletions of the 5′ end of the *FEHIIa* promoter identified a region between –278 to –172 bp that strongly affected cold induction (Fig. 6). PLACE database screening of this region showed the presence of a TACGTGTC motif, also called an ABRE box present in several cold-regulated gene promoters such as *COR15a* (Baker *et al*., 1994), *Rd29A* (Yamaguchi-Shinozaki and Shinozaki, 1994), and *COR6.6* (Wang *et al*., 1995). In addition to cold, the ABRE box is also inducible by exogenous ABA treatment (reviewed in Shinozaki and Yamaguchi-Shinozaki, 2000). Chicory root explants incubated in 30 μM ABA showed a 3-fold increase in reporter gene activity compared with untreated controls (results not shown). These preliminary data support a role for ABA in *FEHIIa* gene regulation. Furthermore, a TGGCCAAC(TT) sequence resides in the same –278 to –172 region that has similarity to another COR promoter, the CRT/DRE element (TGGCCGAC), which is responsible for cold induction through an ABA-independent pathway in *Arabidopsis* (Baker *et al*., 1994; Stockinger *et al*., 1997) and *Brassica napus* (Jiang *et al*., 1996). Kim *et al*. (2001) reported the parallel functioning of both the ABA-dependent (ABRE elements) and ABA-independent (CRT/DRE elements) signals during a cold response in soybean. Recently, it was demonstrated that replacement of TGGCCGAC by TGGCCTAC severely reduced cold-induced expression, while incorporation of two T bases at the end of the motif (which is the case for the *FEHIIa* element) almost doubled cold-related transient promoter activity (Xue, 2002). However, nucleotide-specific substitution analysis of the *FEHIIa* promoter will be necessary to determine the involvement of the ABRE and/or CRT/DRE elements in cold induction in chicory roots.

A role for *FEHIIa* expression in cold protection is far from clear. It is generally recognized that membranes are a primary target for freezing injury in cells (Steponkus, 1984; Oliver *et al*., 2001). Frost-tolerant plants use multiple strategies to survive under adverse environmental conditions including the production of protective proteins and sugars (Hincha *et al*., 1998). Recent reports indicate that oligofructans might have a membrane protective effect during stress conditions (Demel *et al*., 1998; Hincha *et al*., 2002). Interestingly, second phase hardening of winter oat increased FEH and invertase activity in the apoplast and resulted in a rise of apoplastic oligosaccharides and fructans that correlated with an increase in freezing tolerance down to –18 °C (Livingston and Henson, 1998). Thus, it might be postulated that cold induction of *FEHIIa* results in a rise in oligomeric fructan or fructose concentrations in the apoplast responsible for the preservation of plant cellular membranes during freezing stress.

Of further interest, 5′ deletion analysis indicated the presence of negatively regulated *cis*-acting elements in the regions between –933 to –717 and –493 to –278 (Fig. 6). A motif search with the PLACE database revealed the

presence of two S1F boxes at position –789 and –860 that were described by Zhou *et al*. (1992) as down-regulating elements in the nuclear *RPS1* and *RPL21* genes that encode the plastid ribosomal proteins S1 and L21, respectively. However, a motif search of the region between –493 to –278 did not identify any elements known to down-regulate gene expression.

Cold enhanced *FEHIIa* promoter-driven reporter-gene expression 10–14-fold in both etiolated leaves and root explants (Fig. 5). These results suggest that cold-regulated expression of the *FEHIIa* promoter is not tissue-specific and can be induced in leaf and root explants. Moreover, although the relative luc/GUS ratios were much lower compared with cold treatment of etiolated leaf tissue, a 4 °C treatment of green leaf explants also stimulated *FEHIIa* promoter expression (Fig. 5). Interestingly, two partially overlapping REα motifs are positioned immediately downstream of a putative TATA box in the *FEHIIa* promoter. The REα element is found in the promoters of several phytochrome-regulated genes, including the *Lhcb21* gene promoter in *Lemna gibba* (Degenhardt and Tobin, 1996). Of particular interest here is that DNA binding at the REα site of an unidentified nuclear factor was much greater in nuclear extracts from etiolated plants compared with green plants, suggesting that the *Lhcb21* gene is negatively regulated by this transcription factor (Degenhardt and Tobin, 1996). By contrast, the *FEHIIa* gene is up-regulated in etiolated tissue and the position of the two REα elements immediately downstream of the putative TATA box is curious. Although interesting, the REα sequence and others associated with regulation by sucrose (e.g. SURE), hormones (e.g. ASF1, ARF, ERE, GARE, ABRE), etc. require more detailed analysis to determine a role in *FEHIIa* gene expression (Fig. 3).

In summary, this is the first report of the cloning and sequence for a plant fructan 1-exohydrolase gene. Sequence analysis indicated that the intron–exon organization resembles those of vacuolar invertases, including the presence of the mini DPN exon. Furthermore, sequence analysis of the *FEHIIa* promoter indicated the presence of multiple *cis*-acting elements that may be involved in temperature- and tissue-specific control of its expression during plant development. Progressive 5′ deletion analysis of the promoter indicated that the regions between –933 to –717 and –493 to –278 contain down-regulating elements while the region between –278 to –172 is sufficient to confer cold-induced expression. It is proposed that cold-induced expression of *FEHIIa* might play an important role in freezing tolerance of fructan-storing plants.

Acknowledgements

This research was supported by grants from the Fund for Scientific Research (NFSR, Flanders) and the National Science Foundation (NSF, USA).

References

Baker SS, Wilhelm KS, Thomashow MF. 1994. The 5′ region of *Arabidopsis thaliana* cor15a has *cis*-acting elements that confer cold-, drought- and ABA-regulated gene expression. *Plant Molecular Biology* **24,** 701–713.

Bournay AS, Hedley PE, Maddison A, Waugh R, Machray GC. 1996. Exon-skipping induced by cold stress in a potato invertase gene transcript. *Nucleic Acids Research* **24,** 2347–2351.

Cairns AJ, Pollock CJ, Gallagher JA, Harrison J. 2000. Fructans synthesis and regulation. In: Leegood RC, Sharkey TD, von Caemmerer S, eds. *Advances in photosynthesis*, Vol. 9. *Photosynthesis: physiology and metabolism.* Dordrecht: Kluwer Academic Publishers, 301–318.

Claessens G, Van Laere A, De Proft M. 1990. Purification and properties of an inulinase from chicory roots (*Cichorium intybus* L.). *Journal of Plant Physiology* **136,** 35–39.

Degenhardt J, Tobin EM. 1996. A DNA binding activity for one of two closely defined phytochrome regulatory elements in an Lhcb promoter is more abundant in etiolated than in green plants. *The Plant Cell* **8,** 31–41.

Demel RA, Dorrepaal E, Ebskamp MJM, Smeekens JCM, de Kruijff B. 1998. Fructans interact strongly with model membranes. *Biochimica et Biophysica Acta* **1375,** 36–42.

De Roover J, De Winter M, Van Laere A, Timmermans JW, Van den Ende W. 1999. Purification and properties of a second fructan exohydrolase from the roots of *Cichorium intybus*. *Physiologia Plantarum* **106,** 28–34.

Elliott KJ, Butler WO, Dickinson CD, Konno Y, Vedvick TS, Fitzmaurice L, Mirkov TE. 1993. Isolation and characterization of fruit vacuolar invertase genes from two tomato species and temporal differences in mRNA levels during fruit ripening. *Plant Molecular Biology* **21,** 515–524.

Frehner M, Keller F, Wiemken A. 1984. Localization of fructan metabolism in the vacuoles isolated from protoplasts of Jerusalem artichoke (*Helianthus tuberosus* L). *Journal of Plant Physiology* **116,** 197–208.

Fuchs A. 1991. Current and potential food and non-food applications of fructans. Redesigning crop products for biotechnology. *Biochemical Society Transaction* **19,** 555–560.

Goetz M, Roitsch T. 1999. The different pH optima and substrate specificities of extracellular and vacuolar invertases from plants are determined by a single amino acid substitution. *The Plant Journal* **20,** 707–711.

Haouazine-Takvorian N, Tymowska-lalanne Z, Takvorian A, Tregear J, Lejeune B, Lecharny A, Kreis M. 1997. Characterization of two members of the *Arabidopsis thaliana* gene family, *Atβfruct3* and *Atβfruc*t4, coding for vacuolar invertases. *Gene* **197,** 239–251.

Hendry G. 1993. Evolutionary origins and natural functions of fructans, a climatological, biographical and mechanical appraisal. *New Phytologist* **123,** 3–14.

Hincha DK, Hellwege EM, Heyer AG, Crowe JH. 2000. Plant fructans stabilize phosphatidylcholine liposomes during freeze-drying. *European Journal of Biochemistry* **267,** 535–540.

Hincha DK, Oliver AE, Crowe JH. 1998. The effects of chloroplast lipids on the stability of liposomes during freezing and drying. *Biochimica et Biophysica Acta* **1368,** 150–160.

Hincha DK, Zuther E, Hellwege EM, Heyer AG. 2002. Specific effects of fructo- and gluco-oligosaccharides in the preservation of liposomes during drying. *Glycobiology* **12,** 103–110.

Jefferson RA, Kavanagh TA, Bevan MW. 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal* **6,** 3901–3907.

Jiang C, Iu B, Singh J. 1996. Requirement of a CCGAC *cis*-acting

element for cold induction of the *BN115* gene from winter *Brassica napus*. *Plant Molecular Biology* **30,** 679–684.

Kim JC, Lee SH, Cheong YH, *et al*. 2001. A novel cold-inducible zinc finger protein from soybean, SCOF-1, enhances cold tolerance in transgenic plants. *The Plant Journal* **25,** 247–259.

Koehler SM, Matters GM, Nath P, Kemmerer EC, Tucker ML. 1996. The gene promoter for a bean abscission cellulase is ethylene-induced in transgenic tomato and shows high sequence conservation with a soybean abscission cellulase. *Plant Molecular Biology* **31,** 595–606.

Lambowitz AM, Belfort M. 1993. Introns as mobile genetic elements. *Annual Review of Biochemistry* **62,** 587–562.

Levitt J. 1980. Chilling, freezing, and high temperature stress. In: Levitt J, ed. *Responses of plants to environmental stress,* Vol. 1. New York: Academic Press, 3–56.

Lewis DH. 1993. Nomenclature and diagrammatic representation of oligomeric fructans a paper for discussion. *New Phytologist* **124,** 583–594.

Livingston DP, Henson CA. 1998. Apoplastic sugars, fructans, fructan exohydrolase, and invertase in winter oat, responses to second-phase cold hardening. *Plant Physiology* **116,** 403–408.

Lorkovic ZJ, Wieczorek KDA, Lambermom MH, Filipowicz W. 2000. Pre-mRNA splicing in higher plants. *Trends in Plant Science* **5,** 160–167.

Maddison AL, Hedley PE, Meyer RC, Aziz N, Davidson D, Machray GC. 1999. Expression of tandem invertase genes associated with sexual and vegetative growth cycles in potato. *Plant Molecular Biology* **41,** 741–751.

Michiels A, Van den Ende W, Tucker M, Van Laere A. 2003*b*. Chromosomal walking of flanking regions from very short known sequences in GC rich plant genomic DNA. *Plant Molecular Biology Reporter* **21,** 1–8.

Michiels A, Van den Ende W, Tucker M, Van Riet L, Van Laere A. 2003*a*. Extraction of high quality genomic DNA from latex-containing plants. *Analytical Biochemistry* **315,** 85–89.

Oliver AE, Hincha DK, Crowe JH. 2001. Looking beyond sugars: the role of amphiphilic solutes in preventing adventitious reactions in anhydrobiotes at low water content. *Comparative Biochemistry and Physiology* **131,** 515–525.

Ow DW, Wood KV, Deluca M, DeWet JR, Helinski DR, Howel SH. 1986. Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science* **234,** 856–859.

Roberfroid MB, Van Loo JAE, Gibson GR. 1998. The bifidogenic nature of chicory inulin and its hydrolysis products. *Journal of Nutrition* **128,** 11–19.

Shinozaki K, Yamaguchi-Shinozaki K. 2000. Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Current Opinion in Plant Biology* **3,** 217–223.

Simpson CG, Hedley PE, Watters JA, Clark GP, McQuade C, Machray GC, Brown JWS. 2000. Requirements for mini-exon inclusion in potato invertase mRNAs provides evidence for exon-scanning interactions in plants. *RNA* **6,** 422–433.

Simpson CG, Thow G, Clark GP, Jennings SN, Watters JA, Brown JW. 2002. Mutational analysis of a plant branchpoint and polypyrimidine tract required for constitutive splicing of a mini-exon. *RNA* **8,** 47–56.

Steponkus PL. 1984. Role of the plasma membrane in freezing injury and cold acclimation. *Annual Review of Plant Physiology* **35,** 543–584.

Stockinger EJ, Gilmour SJ, Thomashow MF. 1997. *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proceedings of the National Academy of Sciences, USA* **94,** 1035–1040.

Sturm A. 1996. Molecular characterization and functional analysis of sucrose cleaving enzymes in carrot (*Daucus carota* L). *Journal of Experimental Botany* **47,** 1187–1192.

Taper H, Lemort C, Roberfroid MB. 1999. Inhibition of dietary inulin and oligofructose on the growth of transplantable mouse tumor. *Anticancer Research* **18,** 4123–4126.

Tucker M, Whitelaw CA, Lyssenko NN, Nath P. 2002. Functional analysis of regulatory elements in the gene promoter for abscission specific cellulase from bean and isolation, expression and binding affinity of three TGA-type basic leucine zipper transcription factors. *Plant Physiology* **130,** 1487–1496.

Tymowska-Lalanne Z, Kreis M. 1998. The plant invertases, physiology, biochemistry and molecular biology. *Advances in Botanical Research* **28,** 71–117.

Van den Ende W, Michiels A, De Roover J, Van Wonterghem D, Clerens S, Van Laere A. 2001. Defoliation induces 1-FEH II (Fructan 1-Exohydrolase II) in Witloof chicory roots. Cloning and purification of two isoforms (1-FEH IIa and 1-FEH IIb). *Plant Physiology* **126,** 1186–1195.

Van den Ende W, Michiels A, De Roover J, Verhaert P, Van Laere A. 2000. Cloning and functional analysis of chicory root fructan 1-exohydrolase I (1-FEH I), A vacuolar enzyme derived from a cell wall invertase ancestor? Mass fingerprint of the enzyme. *The Plant Journal* **24,** 1–12.

Van den Ende W, Mintiens A, Speleers H, Onuoha AA, Van Laere A. 1996. The metabolism of fructans in roots of *Cichorium intybus* during growth, storage and forcing. *New Phytologist* **132,** 555–563.

Van den Ende W, Van Laere A. 1996. Fructan synthesizing and degrading activities in chicory roots (*Cichorium intybus* L.) during field-growth, storage and forcing. *Journal of Plant Physiology* **149,** 43–50.

Wang H, Datla R, Georges F, Loewen M, Cutler AJ. 1995. Promoters from *kin1* and *cor6.6*, two homologous *Arabidopsis thaliana* genes: transcriptional regulation and gene expression induced by low temperature, ABA, osmoticum, and dehydration. *Plant Molecular Biology* **28,** 605–617.

Warren GJ. 1998. Manipulating freezing tolerance in plants. *Current Biology* **8,** 514–516.

Wiemken A, Frehner M, Keller F, Wagner W. 1986. Fructan metabolism, enzymology and compartmentation. *Current Opinion in Plant Biochemical Physiology* **5,** 17–37.

Wilson R, Michiels A. 2003. Fall herbicide treatments affect carbohydrate content of Canada thistle (*Cirsium arvense*) and dandelion (*Taraxacum officinale*). *Weed Science* **51,** 299–304.

Xu J, Avigne WT, McCarty DR, Koch KE. 1996. A similar dichotomy of sugar modulation and developmental expression affects both paths of sucrose metabolism, evidence from a maize invertase gene family. *The Plant Cell* **8,** 1209–1220.

Xue GP. 2002. An AP2 domain transcription factor HvCBF1 activates expression of cold-responsive genes in barley through interaction with (G/a)(C/t) CGAC motif. *Biochimica et Biophysica Acta* **1577,** 63–72.

Yamaguchi-Shinozaki K, Shinozaki K. 1994. A novel *cis*-acting element in *Arabidopsis* gene is involved in responsiveness to drought, low temperature or high salt stress. *The Plant Cell* **6,** 251–264.

Yamamori A, Onodera S, Kikuchi M, Shiomi N. 2002. Two novel oligosaccharides formed by 1F-fructosyltransferase purified from roots of asparagus (*Asparagus officinalis* L.). *Bioscience, Biotechnology and Biochemistry* **66,** 1419–1422.

Zhou DX, Li YF, Rocipon M, Mache R. 1992. Sequence specific interaction between S1F, a spinach nuclear factor, and an negative *cis*-element conserved in plastid-related genes. *Journal of Biological Chemistry* **267,** 23515–23519.